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97201440.1
Patentanmeldung Nr. Patent application No. Demande de brevet n°

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Anmelde-Nr.: 97201440.1
Anmelde-Nr.: Application no.: 97201440.1
Demanded no.: Demande n°: 97201440.1
Anmelder: Erfinder:
Applicant(s): Demandeur(s):
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NETHERLANDS

Bezeichnung der Erfindung:
Titre de l'invention:
Titre de la invention:
Molekular detection of chromosome aberrations

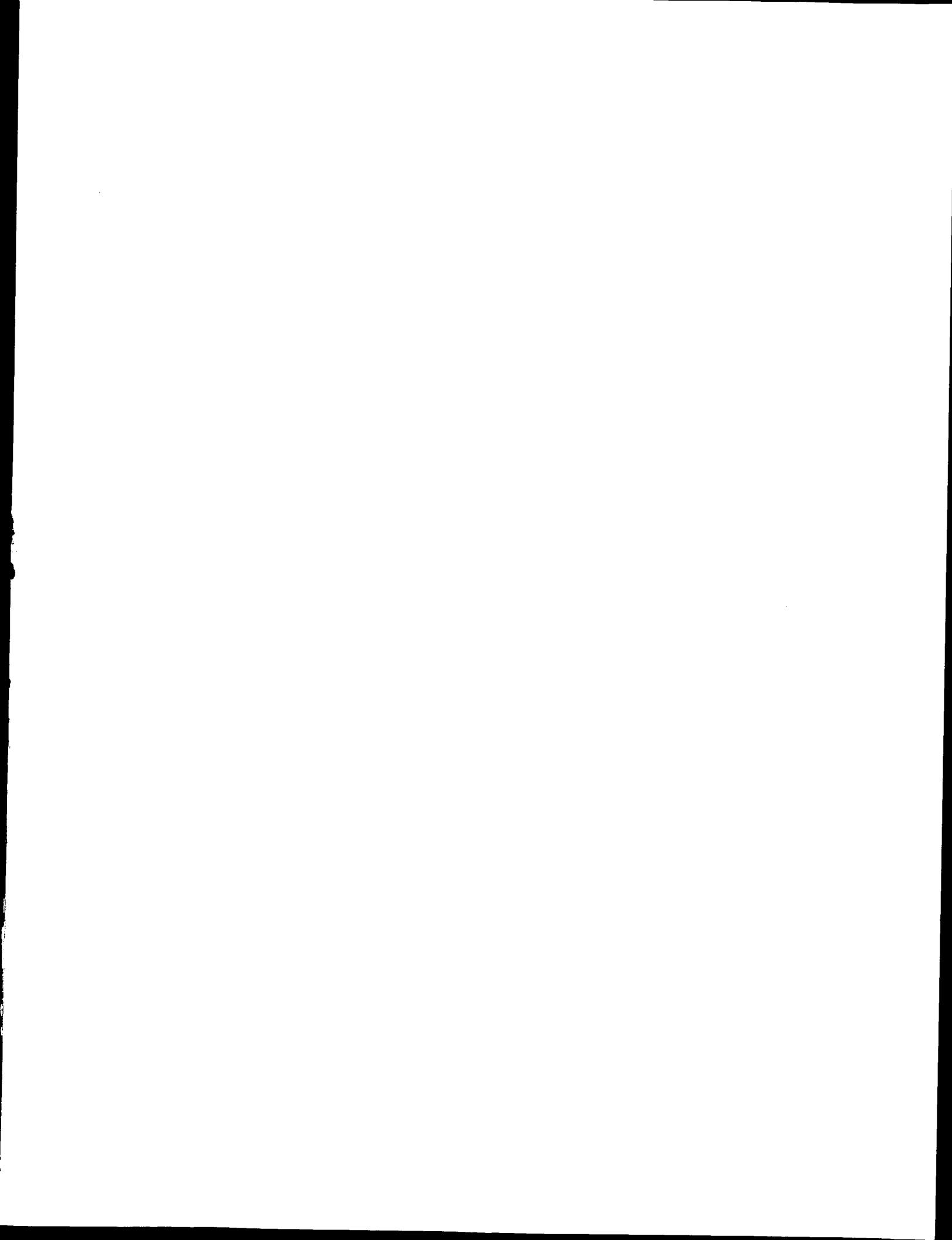
In Anspruch genommene Priorität(en) / Priority(ies) claimed / Priorité(s) revendiquée(s)

Anmelde-Nr.: Tag: Date: File no.: Numéro de dépôt:
Aktenzeichen: Tag: Date: Date de dépôt:
Staats: Pays:

Internationale Patentklassifikation:
Internationale Patentklassifikation:
Internationale Patentklassifikation:
Classement international Patent classification:
Classement international des brevets:
Remarques:
Bemerkungen:

Am Anmeldestag benannte Vertragsstaaten:
Contracting states designated at date of filing: AT/BE/CH/CY/DE/DK/ES/FI/FR/GR/IE/IT/LI/LU/MC/NL/PT/SE/TR

C12Q1/68



Title: Molecular detection of chromosome aberrations.

The invention relates to the field of cytogenetics and the application of genetic diagnostic techniques in pathology and haematology. Specifically, the invention relates to nucleic acid probes that can be used in hybridization techniques for the detection of chromosome aberrations and other gene rearrangements such as immunoglobulin (Ig) and T cell receptor (TCR) genes and acquired diseases such as malignancies. At the basis of the above malignancies lie the fact that all cells of a common clonal origin. Chromosomal aberrations in malignancies stem from rearrangements, inversions, insertions, deletions and other translocations, inversions, insertions, deletions and other aberrations two different chromosomes are involved. In this way, genes, or fragments of genes are removed from the normal chromosome and are located to a recipient chromosome, adjacent to non-related genes or fragments of genes (often oncogenes or proto-oncogenes). Such an aberrant genetic combination can be the cause of a malady.

Often, such rearrangements involving two non-aberrant chromosomes happen in a somewhat established pattern. Breaks occur in either of the two chromosomes at a potential breakpoint or breakpoint cluster region resulting in removal of a gene or gene fragment from one chromosome and subsequent rearranged chromosome where the rearranged fragments are fused in a fusion region.

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2 Detection of chromosome aberrations can be achieved using a wide array of techniques, various of which entail modern biotecnological techniques such as cytogenetic analysis by conventional chromosome banding. Intensive, require skilled personal and are expensive. Automated karyotyping is useful for some diagnostic applications, such as prenatal diagnosis, but is ineffective in analysing the complex chromosomal aberrations such as maldigencies. Furthermore, above techniques require fresh (cultured) cells, which are not always available. Other, more modern, techniques are Southern blotting or other nucleic acid hybridisation techniques or amplification techniques such as PCR, for the detection of well-defined chromosome aberrations such as PCR, for the detection of nucleic acid probes or primers are available. With these techniques, fresh or frozen cells can be used, and sometimes even samples after formalin fixation as long as the nucleic acid sequences to be hybridised or amplified remain intact and accessible. However, even with above modern technology, several disadvantages can be found that hamper the application of these diagnostic techniques in the rapid screening for chromosomal aberrations related to said maligancies.

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Southern blotting lasts 3 to 4 weeks, which is too slow for efficient diagnosis and choice of therapy in malignant neoplasms, and allows only 10-15 kb of nucleic acid sequences to be analysed per probe analysis. PCR, although in essence well-suited for rapid and massive diagnostic testing or even screening, allows only 0.1 to 2 kb of nucleic acid to be analysed per PCR analysis, which greatly hampers rapid screening of vast stretches of chromosomes and breakpoint cluster regions within the chromosomes. An additional disadvantage of PCR is its

of the prognoses and the design of an adequate therapy. Fluorescent *in situ* hybridization techniques (FISH) are less dependent on the complete matching of nucleic acid sequences to get positive diagnostic results. In general FISH employs probe analyses with large, mainly unspecific, varying stringency, with the genes or gene fragments located at both sides of the fusion region in the rearranged chromosome in the malignant cell. Using large probes renders the FISH technique very sensitive. The binding of the colocalizing probes is generally detected either directly or indirectly with fluorochromes and visualized via fluorescence microscopy of a population of cells obtained from the sample. However, even the currently used FISH protocols have inherent disadvantages, these mainly relate to the selection of nucleic acid probes employed in the current FISH of nucleic acids, which can give false-positive results in the protocols, whereas, which can give false-negative results in the testing, let alone in automated testing or screening. Thus far, generally large probes, derived of cosmid clones, YAC clones, or other cloned DNA fragments have been used as probes in FISH. The exact position of these probes is not specific enough to employ standard FISH techniques in massive or rapid diagnostic tests are, albeit sensitive, not specific enough to employ diagnostics of chromosomal aberrations. Hence, the diagnostic protocols, which can give false-positive results in the testing, let alone in automated testing or screening. Thus far, generally large probes, derived of cosmid clones, YAC clones, or other cloned DNA fragments have been used as probes in FISH. The exact position of these probes is not specific enough to employ standard FISH techniques in massive or rapid diagnostic tests are, albeit sensitive, not specific enough to employ diagnostics of chromosomal aberrations. Hence, the diagnostic protocols, which can give false-positive results in the testing, let alone in automated testing or screening.

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 Inherent sensitivity to mismatched primers. Small, normal, and physiologically alterations which can always be present in the nucleic acid sequence of the gene fragment complementarily to the primer hamper the reliable application of PCR and eventually give rise to false-negative results. Especially false-negative results render a PCR-based diagnostic test, albeit very specific, not sensitive enough for reliable diagnosis, and it goes without saying that only a reliable diagnosis of malignancies can contribute to an understanding

The invention further provides a distinct and balanced acid molecule at a genomic distance of no more than 100 kb, but preferably no more than 50 kb. In addition the nucleic acid molecule which hybridise to said balanced part of nucleic acid probes which hybridise to said acid molecules per cell. The nucleic acid probes composed of but generally low-striking conditions to only a few DNA acid probes which hybridise in situ under varying invention provides a distinct and balanced part of nucleic diagnostic tests entailing FISH technology. Furthermore, the invention provides a distinct and balanced part of nucleic acid probes which hybridise in situ, and can i.e. be used in diagnostic tests entailing FISH technology. Furthermore, the invention provides a distinct and balanced part of nucleic acid probes which hybridise in situ under varying conditions per cell. The nucleic acid probes composed of but generally low-striking conditions to only a few DNA acid probes which hybridise in situ under varying conditions per cell.

The development of reliable probes for detection of well-defined or even ill-defined chromosome aberrations in hematological malignancies is described as non-limiting example to illustrate the invention. Such probes can be used for diagnosis and for molecular classification of the involved malignancies. The new probes can be used in diagnostic testing in several types of hematological malignancies with increased sensitivity, specificity, and efficacy of analysis.

Each year world-wide many cases of hematopoietic malignancies are being diagnosed. In the European Union (~375 million inhabitants) this concerns ~98,000 patients per year. The estimated number of patients in the USA (~250 million inhabitants) is ~65,500 per year. The majority of patients with malignant lymphomas, and multiple myelomas. The non-Hodgkin's lymphomas (NHL) form the largest group, representing approximately half of all hematopoietic malignancies. Furthermore, European epidemiological studies show that the incidence of NHL is gradually increasing (~5% per year), which indicates that NHL poses a significant problem throughout the western world. Although the annual number of patients diagnosed with ALL is smaller than for NHL, ALL has a high prevalence in children, representing the most frequent malignancy in childhood.

Lymphoid malignancies consist of a broad range of ~25 different diseases, which differ in clinical presentation, prognosis, and treatment protocols. These diseases have been defined in the recent Revised European American Lymphoid neoplasm (REAL) classification. In this classification the lymphoid malignancies (~90%) and T-cell malignancies (~10%).

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20 Lymphoblastic leukemias (ALL), chronic lymphocytic leukemia, hematological malignancies are of lymphoid origin: acute lymphoblastic leukemias (ALL), chronic lymphocytic leukemia, and multiple myelomas, and most malignant lymphomas. The non-Hodgkin's lymphomas (NHL) form the largest group, representing approximately half of all hematopoietic malignancies. Furthermore, European epidemiological studies show that the incidence of NHL is gradually increasing (~5% per year), which indicates that NHL poses a significant problem throughout the western world. Although the annual number of patients diagnosed with ALL is smaller than for NHL, ALL has a high prevalence in children, representing the most frequent malignancy in childhood.

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heavily skewed in case of a B-cell malignancy. The value of clonality assessment is based on the fact that all cells of a malignancy have a common clonal origin. In lymphoid malignancies this is reflected by the presence of identicality (clonality) rearranged Ig and T cell receptor (TCR) genes: clonal Ig and/or TCR gene rearrangements are found in most (90-95%) immature lymphoid malignancies and virtually all (>98%) mature lymphoid malignancies. Therefore mature lymphoid clonalities of Ig and TCR genes is highly suitable for discrimination between monoclonal (malignant) and polyclonal (reactive) lymphoproliferations. Suspect lymphoproliferations should therefore be subjected to molecular clonality assessment. During the last decade the knowledge about genetic aberrations in hematopoietic malignancies has considerably increased, especially in acute leukemias and NHL. Currently well-established chromosome aberrations are found in 35-40% of ALL and in 30-40% of NHL. These chromosome aberrations can be used as alternative or additional markers for molecular clonality assessment. More importantly, these chromosome aberrations appear to be relevant classification markers, which supplement the currently used morphological and aberrations to be relevant classification markers, which supplement the currently used morphological and clonality assessment. 30

The diagnosis and classification of lymphoid hematopoiesis is mainly based on cytomorphology and maligancies. It is generally based on cytomorphology and hematopoiesis. This is mainly due to the availability of low cytometry and/or lymphohistochemistry. This is mainly due to the availability of lymphohistochemistry and hematopoiesis. Classification of lymphoid maligancies, such as the classification of ALL into pro-B-ALL, common-ALL, pre-B-ALL, and several types of T-ALL. In mature B-cell malignancies and supported by immunophenotypic cloneality assessment via detection of single Ig light chain expression, i.e. the distribution of IgA and IgG positive B-cells, which is 10

From original cytogenetic analysis of chromosomes has demonstrated that several genetic aberrations are associated with a favourable prognosis, such as t(4;11) in pro-B-ALL and t(9;22) in common-ALL. Several treatment protocols have started to use this information for stratification of treatment. Therefore it can be anticipated that rapid and reliable detection of well-defined genetic aberrations will become essential in the diagnosis and management of hematopoietic malignancies.

Several different types of chromosomal aberrations have been identified in ALL and NHL. The chromosome aberrations in pro-B-ALL concern translocations, which result in fusion genes, encoding for fusion proteins with new or modified functions. Examples include the E2A-PBX and BCR-ABL fusion proteins, resulting from t(1;19) and t(9;22), respectively. Another important chromosomal region, the 11q23 region with the MLL gene, is involved in several types of translocations in acute leukemias. In these 11q23 translocations different partner genes are involved, leading to different fusion proteins. One of them is t(4;11), which is observed in ~70% of infant acute leukemias. Many chromosome aberrations in combination with oncogene sequences. These gene sequences in combination with oncogene sequences. These chromosome aberrations do not give rise to fusion proteins, but result in increased or stabilized expression of the involved oncogene, thereby contributing to uncontrolled growth. They occur at relatively high frequency in particular disease categories, such as t(14;18) with involvement of the BCL2 gene in ~90% of follicular lymphomas and t(11;14) with BCL2 gene in ~70% of follicular lymphomas and t(11;14) with involvement of the BCL1/Cyclin D1 gene in ~70% of mantle cell lymphomas.

aberrations. This technique needs the presence of cells in metaphase, which generally requires various cell culture systems, dependent on the type of malignancy. The success rate for obtaining reliable karyograms is highly dependent on the type of malignancy and the experience of the laboratory and ranges from less than 50% to over 90%. Furthermore, some chromosome aberrations can not or hardly be detected by cytogenetic analysis such as T-ALL deletions in T-ALL and (12;21) in precursor-B-ALL. Therefore in case of well-established chromosomal aberrations the laboratory and time-consuming classical cytogenetics is now being replaced by molecular techniques. As said, molecular analysis of genetic aberrations can be performed with Southern blotting, polymerase chain reaction (PCR) techniques, and FISH 10

chromosome aberrations with fusion genes and fusion
 in t(2;5) in large cell anaplastic lymphoma. Despite these
 obvious advantages, the broad application of PCR techniques
 for detection of chromosome aberrations in hematopoietic
 malignancies is hampered by several problems. PCR results
 can be obtained if the DNA or mRNA from formalin-
 fixed paraffin-embedded tissue samples is less optimal than
 antisera, or when primers are mismatching. False-positive
 results might be obtained due to cross-contamination of PCR
 products between samples from different patients; especially
 in case of RT-PCR studies of fusion gene transcripts it might
 be difficult to exclude false-positive results. Finally,
 routine PCR analysis can only be used to study relatively
 small fusion regions of chromosome aberrations (<2 kb). This
 whereas it will be difficult to study large breakpoint or
 fusion regions (>10 kb). This explains the lower
 detectability of chromosome aberrations, and thus again the
 presence of false-negative results, at the DNA level by PCR
 as compared to Southern blotting.

20 A major advantage of FISH techniques as compared to
 cytogenetic analysis, Southern blotting, and PCR analysis is
 that FISH can be performed on interphase nuclei of all kind
 of tissue and cell samples and that there is no need for
 extraction of DNA or RNA. In FISH techniques generally large
 DNA probes (>25 kb) are used, which are located around the
 breakpoint regions that FISH probes can scan much larger regions than
 Southern blot probes or PCR primers. This advantage is
 especially important for detection of breakpoints outside the
 traditional breakpoint cluster regions. Furthermore the use
 of large fluororescently-labeled DNA probes allow direct and

1. The probes of the invention are selected to form a distinct and balanced pair of nucleic acid probes; size of the probes is each within certain limits (e.g. 10-30, or 20-40, or 30-50, or 40-60 kb), so that the intensity of the fluorescence signals of the various probes is comparable 2. In an additional embodiment of the invention the position of the probes constitutes a signal within certain limits (e.g. 10-30, or 20-40, or 30-50, or 40-60 kb), so that the pair is balanced with different fluorescent labels. Thus far, generally cosmid clones, YAC clones, or other cloned DNA fragments have been used without specific selection or modification of these probes. For many of these probes the position in the genome is not precisely known; they often even overlap, with breakpoint clusters regions, and they often contain certain repetitive sequences which cause high background staining. Furthermore, translocations are generally detected by use of two different probes, one for each of the involved chromosomes; these two probes are assumed to colocalize in case of a translocation, but show separate signals if no translocation is present. However, in practice 2 to 4% of normal interphase cells will show false-positive results due to the fact that the two signals colocalize by chance.

For routine applicability of FISH techniques or other aberrations in the diagnosis and classification of hematopoietic malignancies, it is necessary to design probe assays or kits for the detection of chromosome aberrations in the diagnosis and classification of hematopoietic malignancies, it is necessary to design probes of the invention are selected to form a distinct and balanced pair of nucleic acid probes.

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For routine applicability of FISH techniques or other aberrations in the diagnosis and classification of hematopoietic malignancies, it is necessary to design probes of the invention are selected to form a distinct and balanced pair of nucleic acid probes.

rapid visualization of deletions and translocations of the studied gene regions. Application of the latest generation of fluorescence microscopes with multiple fluorochrome filter combinations, CCD camera, and appropriate computer software allow the combined use of multiple FISH probes, which are 5

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EP97201440.1

1. Determined precisely, i.e. no overlap with breakpoint cluster regions, the relevant breakpoints are preferably located within 50 kb or preferably even within 25 kb of either probe, and an additional probe pair has to be designed, if two breakpoint regions of a particular chromosome are separated for more than 30-50 kb depending on the exact position of the probes.

2. In a further embodiment the nucleic acid probes do not contain (major) repetitive sequences, and do not cross-hybridise, which results in high background staining. For this reason the nucleic acid probes composed of several DNA fragments can be tested either on metaphase spreads or with Southern blotting for hybridisation sensitivity and specificity.

3. In a further embodiment the nucleic acid probes do not contain (major) repetitive sequences, and do not cross-hybridise, which results in high background staining. For this reason the nucleic acid probes can alterнатively, or additinally be tested in filter FISH prior to being employed in diagnostic testing, for mapping and checking their relative positions.

4. The nucleic acid probes can alterнатively, or additinally be tested in filter FISH prior to being employed in diagnostic testing, for mapping and checking their relative positions of a chromosome breakpoints becomes easier and more reliable, if two separate probes, labelled with two different

5. It has additinally been found that detection of chromosome breakpoints becomes easier and more reliable, if two separate probes, labelled with two different fluorochromes, constituting said pair are designed around one of the breakpoint regions of a chromosome aberration. This will lead to localisation of a chromosome aberration if no breakpoint is present. However if a breakpoint occurs in the studied breakpoint region, the two different labelled probes will result in two separate signals.

6. In addition, the design of a third probe (labelled with a third fluorochrome) and thus the design of two additinal distinct pairs of probes for the partner gene of the chromosome aberration allows precise identification of the chromosome aberration.

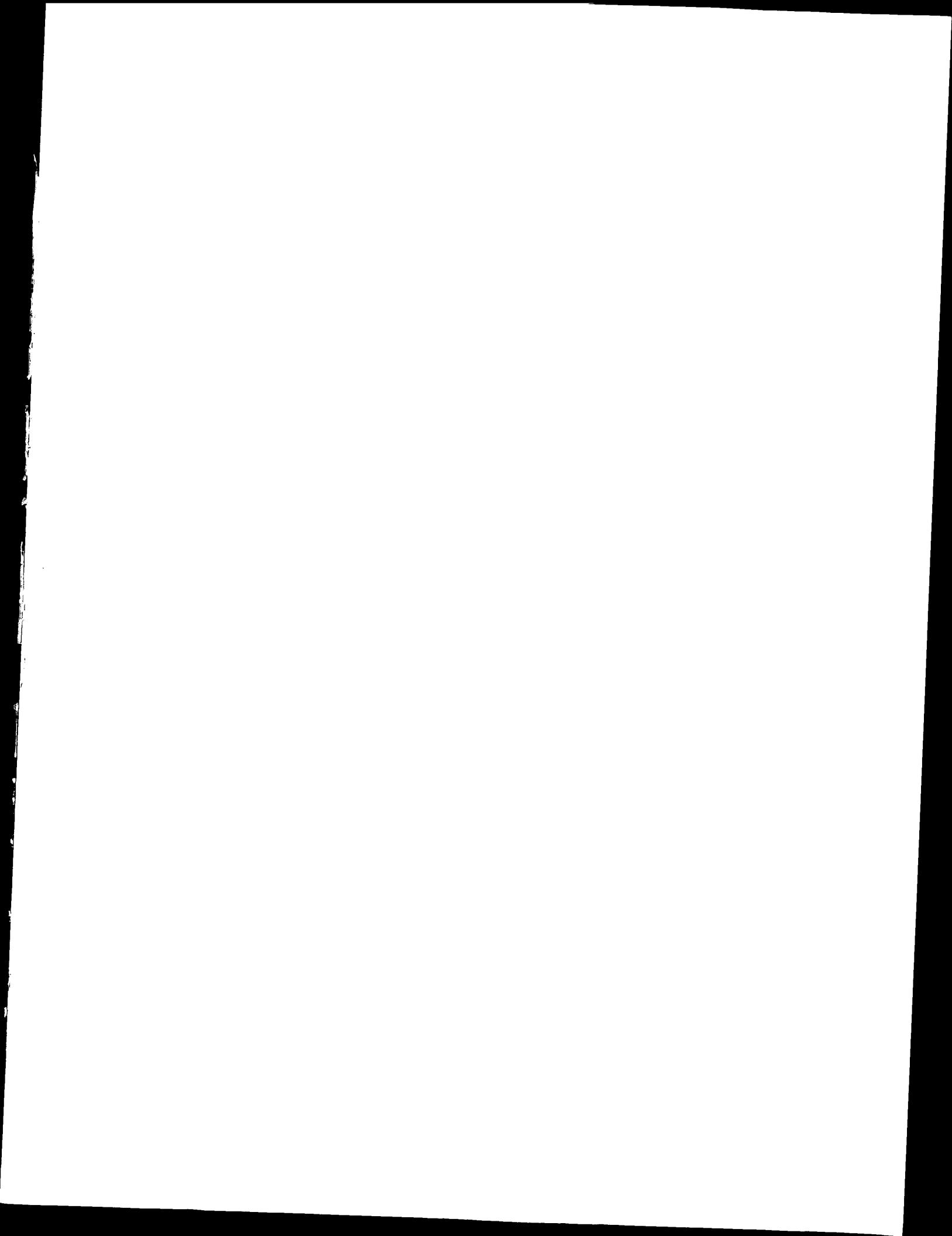
7. Chromosome aberrations found with haemato poetic malignancies are useful for molecular classification of ALL.

The *BCL2* gene consists of only three exons, but these are scattered over a large area. Of these the last exon encodes a large 3', untranslated region (3', UTR). This is one of the two regions in which many of the t (14;18) UTRs are located.

Chromosome aberrations in lymphoid malignancies often involve Ig or TCR genes. Examples include the three types in Burkitt's lymphomas, in which the MYC gene is coupled to Ig heavy chain (IGH), Ig kappa (IGK), or Ig Lambda (IGL) segments, respectively. Another common type of translocation in this category is the t(14;18) (q32;q21) that is observed ~90% of follicular lymphomas, one of the major NHL types. This translocation places the BCL2 gene in rearranged to regions within the IGH locus within or adjacent to the JH gene segments. The result of this chromosome aberration is the overexpression of the BCL2 protein, which plays a role as a survival factor in growth control by inhibiting programmed cell death.

11. Because of its relatively small size, this breakpoint region is easily accessible for molecular detection of translocations. By choosing two distinct -labeled FISH probes in the sequences flanking the breakpoint region, any translocation involving the 11q23 region can be detected on the basis of segregation of the two fluorochromes colocalize when no rearrangement in the *MLL* gene has occurred. Furthermore, the use of a third fluorochrome for probes directed against partner genes enables the identification of the precise type of translocation. This two-step approach of FISH analysis guarantees efficiency and direct detection of all aberrations involving the 11q23 (MLL gene) region in the first step, whereas in the second step the type of 11q23 translocation can be determined.

breakpoints are clustered and is called "major breakpoint region" (mb); the other breakpoint region involved in (14;18) translocations, is located 20-30 kb downstream of the BCL2 locus and is located at the 5' side of the BCL2 locus and is called the "minor cluster region". A third BCL2 breakpoint area, the VCR (variant cluster (mc)). A third BCL2 breakpoint region, is located at the 5' side of the BCL2 locus and is located in (2;18) and (18;22), in which IGR and IGL gene segments are involved in variant translocations, i.e. amongst others involved in variant translocations, i.e. By choosing a set of FISH probes that are located in the partner genes. By choosing a set of FISH probes that are located in the VCR region upstream of the BCL2 locus and downstream of the VCR region, translocations in these regions can be detected upon segregation of the fluorochrome signals. An additional set of FISH probes is designed for the VCR region, since the distance between the VCR region and the other two breakpoint set of FISH probes is too large (~400 kb) to use the same probes. As a second step in all these approaches, FISH probes in the clusters is far too large (~400 kb) to use the same probes. IGH, IGR, and IGL genes are used for identification of the exact type of translocation.



1. A pair of nucleic acid probes of comparable size, each preferably being from 1 to 100 kb, more preferably each being from 1 to 10 kb, or 7 to 15 kb, or 10 to 20 kb, or 10 to 30 kb, or 20 to 40 kb, or 30 to 50 kb, or 40 to 60 kb, or 50 to 70 kb, or 60 to 80 kb, or 70 to 90 kb, or 80 to 100 kb, and 5 kb, or 20 to 40 kb, or 30 to 50 kb, or 40 to 60 kb, or 50 to 70 kb, or 60 to 80 kb, or 70 to 90 kb, or 80 to 100 kb, and each being labeled directly or indirectly with at least one claim 1, flanking a potential break point in a chromosome.

2. A pair of nucleic acid probes according to claim 1, each being labeled directly or indirectly with at least one claim 1, reporting a reporter molecule is selected from the group consisting of enzymes, chromophores, fluorochromes, haptens wherein the reporter molecule is selected from the group consisting of enzymes, chromophores, fluorochromes, haptens 10 of claim 2.

3. A pair of nucleic acid probes according to claim 2 wherein the reporter molecule is selected from the group consisting of enzymes, chromophores, fluorochromes, haptens 15 of claim 1 to 3 characterised in that probes hybridise to a single corresponding nucleic acid molecule.

4. A pair of nucleic acid probes according to any of (such as biotin or digoxigenin).

5. A pair of nucleic acid probes according to claim 4 wherein the corresponding nucleic acid molecule is at least a fragment of a chromosome.

6. A pair of nucleic acid probes according to claim 5 wherein the chromosome is not aberrant.

7. A pair of nucleic acid probes according to any of claims 4 to 6 which hybridise to said nucleic acid molecule 20 at a genomic distance of no more than 100 kb, but preferably claims 4 to 6 which hybridise to any of the claims 1 to 7 which hybridise in situ.

8. A pair of nucleic acid probes according to any of the claims 1 to 7 which hybridise in situ.

9. A pair of nucleic acid probes according to any of the claims 1 to 8 which hybridise in situ under low-temperature conditions to only a few linear DNA molecules per cell.

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Claims

10. Use of a pair of nucleic acid probes according to any of claims 1 to 9 for the detection of a nucleic acid molecule comprising a chromosome aberration.

11. Use of a pair of nucleic acid probes according to any of claims 1 to 9 for the detection of cells comprising a chromosome aberration.

12. Use of a pair of nucleic acid probes according to any of claims 1 to 9 for the detection of a disease caused by a chromosome aberration.

13. Use of a pair of nucleic acid probes according to any of claims 10 to 12 wherein the chromosome aberration is related to a maligancy.

14. Use of a pair of nucleic acid probes according to any of claims 10 to 12 wherein the chromosome aberration is related to a hematopoietic maligancy.

15. A diagnostic kit comprising at least a pair of nucleic acid probes according to any of claims 1 to 9.

The invention relates to the field of cytogenetics and the application of genetic diagnostic techniques in pathology and hematology. Specifically, the invention relates to nucleic acid probes that can be used in hybridization techniques for the detection of chromosomal aberrations and other gene rearrangements such as immunoglobulin (Ig) and T cell receptor (TCR) gene rearrangements. The probes provided by the invention are a distinct and balanced pair of probes of comparable size each preferably being from 1 to 100 kb, or smaller, and flanking a potential breakpoint in a chromosome.

ABSTRACT

